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Allelopathic inhibition of spruce germination

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Abstract

Subalpine spruce (*Picea abies*) forests in the northern Alps are facing serious problems in natural regeneration. This paper presents the results of research carried out into the possible existence of allelopathic mechanisms operating within these stands.

In vitro experiments have demonstrated the sensitivity of the germination phase to hydrolysates produced by fronds of *Athyrium filix-femina*, a very common species found in tall-forb-understory spruce stands and by leaves of *Vaccinium myrtillus*, typical of bilberry-understory stands. Spruce auto-intoxication (from needle hydrolysates) is also brought to light. Humic solutions derived from bilberry-understory (mor) and tall-forb-understory (mull-moder) stands only cause delayed germination.

Biochemical analysis of leaf hydrolysates and humic solutions was carried out. The molecules responsible for the observed inhibition could be phenolic acids (catechol, p-hydroxyacetophenone and p-hydroxybenzoic acid), or flavonols and proanthocyans.

Keywords: *Athyrium filix-femina*, *Picea abies*, *Vaccinium myrtillus*, allelopathy, germination inhibitors, humus, natural regeneration, phenolic compounds.

Résumé

Les forêts sulbalpines d'épicéa rencontrent de graves problèmes de régénération naturelle dans les Alpes du Nord. De multiples causes concourrent à expliquer cette déficience et, parmi elles, l'hypothèse d'une inhibition allélopathique de la germination de l'épicéa est testée.

Des expérimentations *in vitro* démontrent la sensibilité de la phase de germination à des hydrolysats provenant de frondes d'*Athyrium filix-femina*, espèce très répandue dans les pessières à mégaphorbiaie et de feuilles de *Vaccinium myrtillus*, caractéristique des peuplements à myrtille. Une auto-intoxication de l'épicéa (hydrolysats provenant des aiguilles) est aussi mise en évidence. Les solutions humiques issues des peuplements à myrtille (mor) et mégaphorbiaie (mull-moder) occasionnent un retard de germination d'une dizaine de jours.

L'analyse biochimique des hydrolysats foliaires et solutions humiques a été réalisée. Les molécules susceptibles d'être responsables des inhibitions observées pourraient être des acides phénoliques (catéchol, p-hydroxyacétophénone et acide p-hydroxybenzoïque essentiellement), ainsi que des flavonols et proanthocyanes.

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INTRODUCTION

In the moist, northern Alps, spruce (*Picea abies* (L.) Karst.) occupies 65 to 80% of the coniferous forests. In the subalpine, this species forms extensive, virtually monospecific stands typified by deficiency in natural regeneration (André *et al.*, 1986). Although Roques and Trosset (1986) have demonstrated severe parasitism of the seed, this factor alone cannot explain the regenerative failure of subalpine spruce stands.

Allelopathic phenomena may have a considerable effect on forest ecosystem dynamics, and some researchers believe them to be involved in alternation of species. For example, Moreau and Poly (1967) have described the fir-beech-spruce succession as being the result of auto-intoxication by each species' own root exudates, and Drapier (1983) has shown the inhibiting action of fresh fir bedding on its own seeds.

Consequently, the allelopathic hypothesis may explain the difficulties in regeneration encountered by subalpine spruce. Some understory species develop well and may produce secondary compounds that have an impact on spruce regeneration. Among the possible phytotoxic agents, *Vaccinium myrtillus* L. (leaves) and *Adenostyles alliariae* (L.) Roth (fronds) were chosen due to their typically extensive cover in bilberry-undergrowth stands and top grass formations. The possibility of spruce auto-intoxication (needles) is investigated. Similarly, since metabolites manufactured by the above-ground organs may also undergo transformations when mixed with the humus into which they fall, the phytotoxic capacities of mor (bilberry-undergrowth stand) and mull-moder (tall-forb-undergrowth stand) humic solutions were also tested.

MATERIAL AND METHODS

Plant collection and biochemical analysis of the potentially phytotoxic solutions

Collection of the plant material and humic solutions was done in lots D and K of the common woodland in Cohennoz, Savoie, France. As the biochemical composition of such solutions can vary through the year (Kuiters, 1987), samples were taken from mid-June to the end of August, i.e. during the most intense growing period in high-altitude spruce stands.

Foliage of *V. myrtillus*, *A. filix-femina* and *P. abies* was collected for leaf hydrolysates from a variety of areas within the lots. It was air-dried at ambient temperature for three weeks, then ground and mixed to produce a very fine powder. Aqueous extraction of the powder was accomplished by means of 12 hours agitation in demineralized water at a concentration equivalent to 1% by dry weight at + 4°C. Extraction was followed by filtration at + 4°C, and sterilization of the filtrate on a 0.22 μm membrane.

The humic solutions were collected after rainy periods using gutters derived from a system devised by Dambrine (1985) for upper mountain soils. Following rains, the solutions were collected as quickly as possible, and then sterilized on a 0.22 μm membrane, in the same way as the leaf extracts.

For hydrolisis, 50 ml aqueous leaf extract was put into 100 ml 2N hydrochloric acid in a conical flask, in a water bath at 100°C. The humic-solution ratio was also 50: 100. After 40 min, the contents were cooled under cold running water.

For the global dosage of the anthocyans, the mother solution was successively extracted with 60, 60 and 40 ml ethyl ether. The anthocyans remained in the red, acid hypophase, and the more lipophilic

flavones, flavonols and a proportion of the phenolic acids passed into the ethereal epiphase. The volume of aqueous solution was recorded and then, following filtration on sintered glass, the spectrophotometric composition determined immediately by "scanning" between 400 and 600 nm.

Determination of flavonols quantities was based upon the specific chelation of these compounds by aluminium. The ethereal epiphase is dry-evaporated under a fume hood, and then taken up in 20 ml ethanol distilled at 95°C. The blank solution contains 0.1 ml of the alcoholic flavonols solution to be dosed in the presence of 5 ml ethanol distilled at 95°C. The reactant solution contains 0.1 ml alcoholic flavonols solution and 5 ml solution of 1% aluminium chloride ($AlCl_3$) in ethanol. After 10 min of reaction, a yellow colour appears and the differential spectrum is determined between 350 and 550 nm. The presence of flavones can be detected by a maximum absorption peak at 390 to 415 nm and flavonols at 420 to 440 nm.

The high-performance liquid chromatography of phenolic acids and anthocyans was performed using a "Waters" apparatus, "5000 A" pump and "M 440" detector. For the phenolic acids, the liquid phase is a mixture of solvent A: 0.5% acetic acid and solvent B: 0.5% acetic acid and 99.5% acetonitrile. A linear gradient system ensures optimum separation. The concentration of B in A rises from 0 to 35% in 35 min. For the anthocyans, a methanol/water/acetic-acid (45/55/05) ternary solution is used, in isocratic conditions. In both cases a μ Bondapack C18 column (Waters) was used. Spectrophotometric detection of the phenols is effected by multidetection at 250, 260, 270, 280, 290, 300, 310, 320 and 330 nm; the flavonols, at 365 nm. Compounds content is estimated by comparing the height of the corresponding peaks relative to standards.

Spruce germination tests

The seeds (source: Hautes Chaînes du Jura listed stands) were supplied by the Joux seed kilns. Due to seed shortage since 1981, seed of the stands studied were unavailable.

In order to avoid any possible inhibition from toxins produced by bacteria or fungi (Tinnin & Kirkpatrick, 1985), the operations were carried out under axenic conditions. Seeds were disinfected by immersion for one hour in sterile, demineralized water and 110 vol. hydrogen peroxide (70: 30 volume to volume), and then rinsed 5 times in sterile, demineralized water. Twenty seeds were then placed in a sterile Petri dish on a filter paper that had been impregnated with 10 ml leaf hydrolysate or humic solution sterilized by filtration through a 0.22 μm membrane. Ten replicates per extract were run, with control dishes containing 10 ml sterile, demineralized water. The dishes were placed in a thermostatically-controlled light-box (photophile phase: 16 hrs at 24°C; skotophile phase: 8 hrs at 14°C). Each day, the number of germinated seeds was counted (rupture of seed coat and emergence of radicle), for 15 days after which no further seeds germination occurred.

Given the relatively low number of replications for each test, non-parametric tests were used (Siegel, 1956). Since the treatments were independent of the controls, the Mann-Whitney U-test was used. Given the independent nature of the controls with respect to the treatments, we calculated an index which showed the effects of the phytotoxic solutions on germination (Williamson & Richardson, 1988). The "Response Index" (RI) is as follows:

when germination of the Treatment (T) is higher than that the Control (C), $RI = 1 - (C/T)$

when germination of the Treatment is lower than that of the Control, then $RI = (T/C) - 1$

Therefore, $-1 \leqq RI \leqq +1$ with: $RI > 0$ if the treatment stimulates germination

$RI = 0$ if the effect of the treatment is nil

$RI < 0$ if the treatment inhibits germination

RESULTS

Biochemical comparison of leaf hydrolysates and humic solutions

The molecules identified during the analyses (table I) must be viewed as being descriptive for a given moment, i.e. the beginning of summer. Compounds identical to those identified by GALLET (1988) for the spruce and bilberry, and by VOIRIN (1970) for the female fern have been found in the aqueous extracts analysed here. Determination and identification of the proanthocyans and flavonols proved positive only for the plant extracts. The humic solutions do not appear to contain these two groups of molecules. A non-identified compound does appear in the two solutions' spectrophotometric profiles, however, with a maximum absorbance of 360 nm.

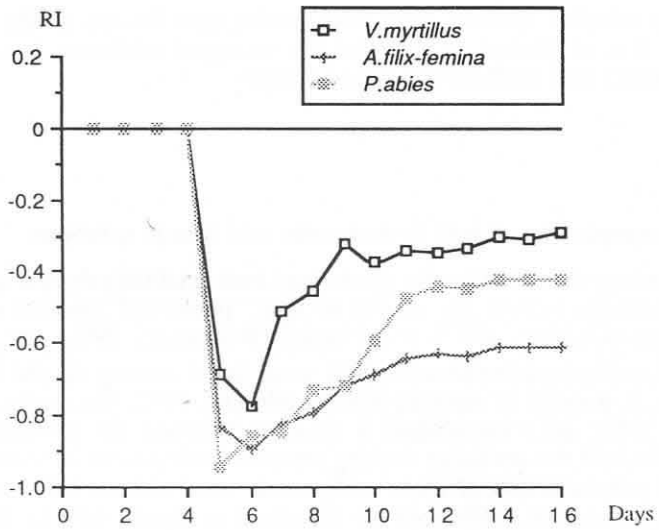
The p-hydroxyacetophenone found in the two humic solutions could come from the spruce since analyses performed on the other species did not bring it to light, and ESTERBAUER *et al.* (1975) have already identified it in spruce.

Allelopathic inhibition of spruce germination

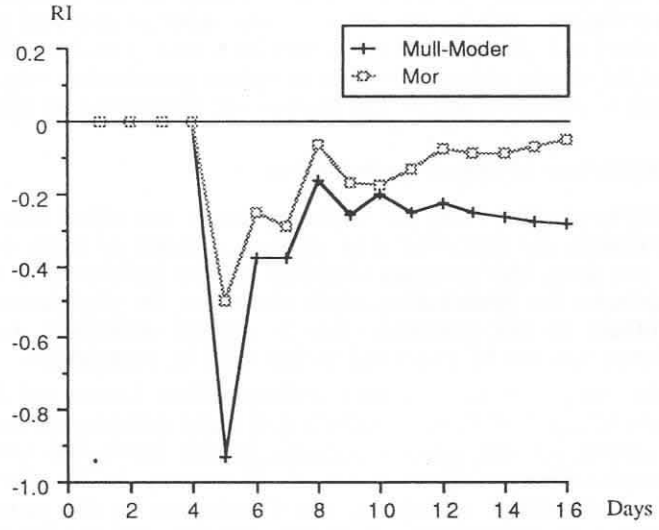
All the plant extracts caused significant delay and inhibition in terms of germination (fig. 1). The effect of the humic solutions was less extreme and is

TABLE I. – *Biochemical composition of leaf hydrolysates (expressed as mg.g^{-1} D.W.) and humic solutions (expressed as 10^{-5} M.l^{-1}). (1): an unknown, non-flavonic, compound is detected.*

	Proanthocyans	Flavonoids	Phenolic Acids
Athyrium filix-femina (mg g^{-1} DW)	1.14	kaempferol: 0.9 quercetin: 0.6	catechol: 95.2 p-OHbenzoic: 3.25 protocatechic: 1.2 cafeic: 0.9 vanillic: 0.88 p-coumaric: 0.48
Vaccinium myrtillus (mg g^{-1} DW)	0.23	kaempferol: 0.45 quercetin: 0.03	catechol: 24.8 p-OHbenzoic: 1.2 protocatechic: 0.43 cafeic: 0.4 vanillic: 0.3 p-coumaric: 0.21
Picea abies (mg g^{-1} DW)	4.32	kaempferol: 0.99 quercetin: 0.56 isorhamnetin: 0.56 myricetin: 0.23	p-OHacetophenone: 103.2 catechol: 94.7 p-OHbenzoic: 47.4 p-coumaric: 5.76
MULL-MODER (10^{-5} Mol l^{-1})	none	none (1)	p-OHacetophenone: 1.09 catechol: 0.11 p-OHbenzoic: 0.03 protocatechic: traces
MOR (10^{-5} Mol l^{-1})	none	none (1)	p-OHacetophenone: 5.83 catechol: 0.35 p-OHbenzoic: 0.28



1-a: leaf hydrolysates



1-b: humic solutions

FIG. 1. – Response Index (RI) of spruce seed germination in the presence of leaf hydrosylates (1–a) and humic solutions (1–b).

particularly expressed by a delay in germination for the mor solution (see RI from 5th to 12th day, figure 1-b). Delay and inhibition of germination result from the

presence of a solution derived from mull-moder type humus in the spruce seed environment. It is of interest to note that the strongest inhibition is caused by the fern frond extract and mull-moder type solution.

DISCUSSION

Biochemical comparison of leaf hydrolysates and humic solutions

Compounds synthesized by the plants vary both qualitatively and quantitatively according to various factors: the quality of light "perceived" (Koeppe *et al.*, 1970), hydric nutrients (Gilmore, 1977), plant health (Woodhead, 1981), etc.

Catechol and p-hydroxybenzoic acid were listed among all the hydrolysates and solutions. A number of authors (Chu & Muller, 1972; Ballester *et al.*, 1982; Kil & Yim, 1983; etc.) established a relation between the phenolic nature of these molecules and the resulting toxicity towards germination or seedling growth. However, the concentration of these compounds must also be taken into account. For example, Blum *et al.* (1984) set the threshold at around 10^{-4} to 10^{-5} M below which the effect is null (or even, occasionally stimulating). Even at 10^{-6} M in this study, a delay in germination was observed, due to catechol and p-hydroxybenzoic acid in the humic solution.

Based on the quantities of proanthocyans, flavonols and phenols determined in the leaf hydrolysates and humic solutions, the order of potential phytotoxicity would be: *P. abies* > *A. filix-femina* > *V. myrtillus* > mor > mull-moder. This scale is corroborated by results obtained in tests of spruce germination (fig. 1): *P. abies* is autotoxic and *V. myrtillus* and *A. filix-femina* are phytotoxic for spruce.

Allelopathic inhibition of spruce germination

Berrie (1984) considers that the inhibiting factor can affect seed metabolism directly, or indirectly by depriving it of oxygen, perhaps as result of phytotoxin oxidation. In our tests, the necroses observed on the radicles lead one to think of two mechanisms: the germinating seeds encounter the phytotoxins and suffer accordingly; others do not germinate due to oxygen deficiency. Unfortunately, oxygen deficiency can not be measured in this kind of experiment.

Phytotoxins may also have another indirect effect: Lynch and Pryn (1977), and Harper and Lynch (1979) have shown that these compounds may constitute a source of carbon for the micro-organisms in the immediate vicinity of the seed ("spermosphere" sensu Dommergues & Mangenot, 1970). The consequent intensification of microbial activity causes a reduction in the concentration of oxygen available, and competition sets in between the seed and the neighbouring micro-organisms. As our germinating experiments were conducted in axenic conditions, the inhibition observed is likely to have been direct.

These *in vitro* results suggest that spruce germination could be sensitive to allelopathy. Natural conditions are, however, more complicated than glasshouse bioassays. As suggested by Stowe (1979), field experiments (small-scale distribution of *P. abies* seedlings, *V. myrtillus* and *A. filix-femina*) are necessary before any final comment is made on allelopathic interference in spruce regeneration.

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